Theriogenology 96 (2017) 69–75

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Quality and fertility of frozen ovine spermatozoa from epididymides stored at room temperature (18–25 °C) for up to 48 h post mortem

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ARTICLE INFO

Article history:
Received 13 January 2017
Received in revised form
28 March 2017
Accepted 1 April 2017
Available online 4 April 2017

Keywords:
Epididymis
Seminal plasma
CASA

ABSTRACT

This study investigates the effect of time of storage of epididymides at room temperature and the addition of 20% of seminal plasma to the cryopreservation extender, on post thaw quality and fertility of ovine spermatozoa collected from the cauda epididymis. Spermatic kinetics, integrity and the stability of plasma membrane, damage to the acrosome and fertility following laparoscopic artificial insemination were evaluated in samples collected in an artificial vagina (AV) and from epididymides stored at room temperature for zero (G0), six (G6), twelve (G12), twenty-four (G24) and forty-eight (G48) hours post mortem. There were no significant differences in spermatic parameters between the methods of sample collection, except for progressive motility and velocity according to the straight path(VSL). G48 samples had significant lower total motility(TM), progressive motility(PM), kinetic parameters, viability and acrosomal integrity. Pregnancy rate after insemination was similar for samples collected using AV, and the G0, G6, G12 and G24 samples. In conclusion, ovine epididymides can be exposed to room temperature, for up to 24 h post mortem, with no effect on viability and fertility of cryopreserved seminal samples. The addition of seminal plasma to the cryopreservation extender had no effect on spermatozoa quality nor fertility.

1. Introduction

In modern livestock breeding the preservation of gametes after the death of a genetically valuable animal can be important. Besides that the preservation of gametes post mortem is an important tool to form a genetic bank for wild animals, especially for endangered species [1]. Authors have report recovery of viable spermatozoa from the cauda epididymis of sheep [1], goats [2], bulls [3], stallions [4], dogs and cats [5] and red deer [6] when the epididymides were kept at 5 °C. However, the maximum period for which epididymis can be kept at room temperature post mortem and viable spermatozoa can still be collected for freezing whilst maintaining their fertilization potential is not well understood. Spermatozoa from cauda epididymides are susceptible to environmental temperature variation [1,7–9]. Some researchers have investigated the maximum sperm viability period when cauda epididymides were kept refrigerated at 5 °C [3,10]; and also it has studied the effect of extender osmolarity and glycerol concentration on post-thawing quality of epididymal spermatozoa [11]. However, in reality, most dead livestock and wild animals are discovered after lying at ambient temperatures for some time.

It has been reported that some components of seminal plasma can prevent or reverse damage caused by temperature variation in ovine spermatozoa [12]. Spermatozoa collected from epididymides lack the protection of seminal plasma. Addition of seminal plasma to spermatozoa collected from epididymides seems to stimulate motility initially [13] and have beneficial effects after thawing [14]. When seminal plasma is added to swine epididymal spermatozoa extender after thawing some authors observed an increase in gestation rate and litter size [15]. To the authors' knowledge there are no reports of the fertility potential of ovine epididymal spermatozoa cryopreserved with the addition of seminal plasma.

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http://dx.doi.org/10.1016/j.theriogenology.2017.04.001
0093-691X/

The objectives of this study were: 1. To evaluate the viability and fertility of ovine epididymal spermatozoa after thawing when epididymides were kept at room temperature (18–25 °C) for 48 h after death using as control semen collected with an artificial vagina; 2. To evaluate the addition of 20% seminal plasma to the cryopreservation extender of ovine semen collected with an AV or from the cauda epididymis.

2. Materials and methods

2.1. Experiment design

This project was approved by the Ethics Committee on Animal Use (CEUA) of the agricultural sciences sector of the Federal University of Paraná – Brazil, protocol number 52/2016.

Ten cross breed rams, aged between 24 and 48 months, were used. The animals remained in an intensive system, with access to pasture during the day and were confined overnight. They received food supplementation with corn, soybean meal and a mixture of minerals and water *ad libidum*. Inclusion criteria were the rams that, although semen collection were performed in non-breeding season, presented the following parameters in fresh semen subjective evaluation: TM higher than 80%, PM higher than 70%, morphological defects lower than 15% and membrane functionality (hiposmotic swelling test) higher than 90%.

With the use of a sheep as a dummy, semen collection was performed using the artificial vagina (AV) method. The internal temperature of the artificial vagina was 40 °C–43 °C. AV collections were performed twice a week for two months in summer (January and February), and the rams were subsequently slaughtered. A section was made in the region of the spermatic funiculum for excision of the scrotum, testis and epididymis. Scrotum, testis and epididymis were transported to the laboratory in a styrofoam box at room temperature (18–25 °C). In the laboratory scrotum, testis and epididymis were removed from the styrofoam box and allocated in empty Becker dishes maintained on room temperature and covered with paper towel until the end of the period of exposure to room temperature.

Scrotum, testis and epididymis were randomly divided into five groups, corresponding to the period of exposure to room temperature (18–25 °C): zero hours (G0), 6 h (G6), 12 h (G12), 24 h (G24) and 48 h (G48). Each group consisted of four epididymides from four different rams.

2.2. Post mortem spermatozoa recovery

After the period of exposure to room temperature a scalpel blade was used to incise the scrotal skin and extract testes and epididymides [16]. Testes and epididymides were washed with 0.09% sodium chloride solution warmed to 35 °C. The tail of the epididymis was divided into warmed petri dishes and maintained at 35 °C. Using anatomical tweezers, scissors and a scalpel blade, superficial blood vessels were dissected to minimize blood contamination of semen. The cauda epididymis was sectioned and light pressure was applied to expel semen from the tubules [1]. After the first cut the role tissue of cauda epididymis was sliced to maximize spermatozoa recovery. The cauda epididymis was then lavaged with 2 mL of control media (CM) warmed to 35 °C. After 5 min a pipette was used to collect the diluted semen from the Petri dish and the sample was placed in a conical tube (Falcon BD) [17].

2.3. Cryopreservation

The control medium (CM) used in the cryopreservation process consisted of 75 mL mother solution (200 mL distilled water, 1.4 g glycine, 2.97 g sodium citrate, 3 g fructose, 0.004 g amikacin), 15 mL skim milk, 5 g egg yolk and 4.6 distilled water and 4% glycerol [18]. The SP medium was formed with addition of 20% seminal plasma to the CM. The seminal plasma was from the same rams from which semen had been collected using an artificial vagina in summer (January and February). The seminal plasma was obtained, and extender with seminal plasma was produced following the methodology of López-Pérez and Pérez-Clariget [19].

Dose dilution with CM and SP extenders was performed to obtain a concentration of 400 million spermatozoa per mL. Inseminating doses were packed in 0.25 mL French straws with 100 million spermatozoa per dose. The doses were frozen in automated equipment (TK 3000®, TK Congelações, Brazil) with a standard ovine freezing curve "S3P2" that decreased from ambient temperature to 5 °C in ratio of 0.5 °C per minute, straws were stabilized at 5 °C for 2 h, after that the temperature decreased 15 °C per minute until −80 °C and in ratio of 10 °C per minute until −120 °C then straws were submerged at liquid nitrogen. Samples were thawed in a water bath at 40 °C for 20s. Sperm quality parameters were evaluated immediately after thawing.

2.4. Spermatozoa evaluation

2.4.1. Fresh analysis

Before freezing analysis were performed in a smaller laboratory next to slaughterhouse and far from the main laboratory with CASA system. TM and PM were evaluated by optical microscopy(Coleman, N 107, Brazil). A drop of semen diluted to concentration of 400×10^6 sperm per mL on CM or SP was deposited between slide and cover slip warmed at 37 °C and examined at 400× magnification. All the analysis were performed by the same person. Sperm morphology was assessed by a differential count of 200 cells on slides prepared with swabs of semen diluted and stained according to the method of Cerovsky (Cerovsky, 1976).

2.4.2. Post thaw motility parameters

Motility was assessed using a computer-assisted sperm analysis system (CASA) (Hamilton Thorn Motility Analyser – HTMA –IVOS 12 – Hamilton Research – Beverly, MA, USA). For evaluation of the sperm kinematic, 30 μL thawed sperm was diluted in 300 μL CM and warmed to 37 °C. Then, 6 μL of diluted semen was deposited into the Makler chamber (Makler Counting Chamber – Selfi-Medical, Haifa, Israel). One frame of the chamber was chosen by the examiner and two other frames were randomly chosen by the equipment and analyzed for total motility (TM,%), progressive motility (PM,%), velocity according to the smoothed path (VAP, μm/s), velocity according to the straight path (VSL, m/s), velocity according to the actual path (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), head beat-cross frequency (BCF, Hz), straightness (STR, %) and linearity index (LIN, %). The setup used was based on the manufacturer's recommendations, (HAMILTON THORNE SETUP FOR IVOS-12.3). The cell size was 5 pixels, cell intensity 55, VAP 75 μ/s, STR 80%, VAP cutoff 21.9 μ/s, VSL cutoff 6.0 μ/s, minimum static intensity gates 0.25, maximum static intensity gates 1.5, minimum static size gates 0.6 and maximum static size gates 8.0, minimum elongation gates 0 and maximum elongation gates 95. Magnification calibration was of 1.95, video source frequency of 60 Hz, 2400 light intensity, low photometer 73 and high photometer 125.

2.4.3. Flow cytometry post thaw

Flow cytometry sperm evaluation was performed in a BD LSR Fortessa (Becton Dickinson, Mountain View, CA, USA) equipped with lasers: blue 488-nm, 100 mW, red 640-nm, 40 mW and violet 405-nm, 100 mW. After analysis, the data were evaluated by

software from the same manufacturer BD FACSDiva ™ software v6.1.

2.4.3.1. Sperm viability and acrosome membrane integrity. For measurement of plasma membrane integrity and acrosome reaction a pool of probes consisting of Pisum sativum agglutinin conjugated to fluorescein isothiocyanate (FITC-PSA; L-0770, Sigma), Propidium Iodide (IP; P4170, Sigma) and Hoechst 33342 (H324; 14533, Sigma) were used as described by Ref. [20]. Samples were diluted in 200 μL of TALP extender (final concentration of 5×10^6 spermatozoa/mL) and incubated for 8 min at 37 °C with 5 μL IP (50 μg/mL), 10 μL H324 (100 μg/mL) and 0.25 μL FITC-PSA (1 mg/mL). In all flow cytometer analysis, Hoechst 33342 fluorescence was used to gate out the non-sperm events. Four different categories were obtained: category 1 (CAT 1, FIT-PSA-/IP +) damaged plasma membrane and unreacted acrosomal membrane; Category 2 (CAT 2, FIT-PSA +/IP-) intact plasma membrane and reacted acrosomal membrane; Category 3 (CAT 3, FIT-PSA-/IP+) intact plasma membrane and unreacted acrosomal membrane; (CAT 4, FIT-PSA + /IP +) damaged plasma membrane and reacted acrosomal membrane [21]. The percentage of spermatozoa with damaged acrosomes was calculated as the sum of CAT 2 and CAT 4.

2.4.3.2. Sperm viability and membrane stability. For assessment of sperm viability and cell membrane stability a combination of merocyanine 540 (M540), YO-PRO1 and H324 probes was used. Samples were diluted in 100 μL of TALP extender (final concentration of 5×10^6 spermatozoa/mL) and incubated for 8 min at 37 °C with 1 μL YoPRO1 (25 μM in DMSO), 2.6 μL M540(1 mM in DMSO) and 10 μL H324 (100 μg/mL). Spermatozoa were classified as: Category 1 (CAT 1 YOPRO1 + /M540-) dead; Category 2 (CAT 2 YOPRO1-/M540-), viable with plasma membrane stability; Category 3 (CAT3, YOPRO1-/M540 +), with viable instability of plasma membrane [22]. The percentage of unstable membrane spermatozoa were equal to CAT3 percentage. The sum of CAT 1 and CAT 2 were used to calculate the percentage of viable spermatozoa.

2.5. Artificial insemination

Estrus was synchronized in 100 cross breed sheep (74 sheep aged between 9 and 15 months and 26 sheep aged between 15 and 36 months) using intravaginal implants impregnated with 60 mg of medroxyprogesterone acetate (Progespon®, Zoetis, United States) for a period of 14 days at the end of breeding season (July). Five ewes lost their implants and were excluded before the artificial insemination. On the day of implant removal 500UI of eCG (Novormon®, Zoetis, United States) was administrated [23]. The ewes were randomly assigned to the groups: AVCM:13 sheep, AVSP:13 sheep, G0CM: 7 sheep, G0SP: 10 sheep, G6CM: 7 sheep, G6SP: 7 sheep, G12CM: 8 sheep, G12SP: 10 sheep, G24CM: 10 sheep and G24SP: 10 sheep. Samples from G48 were not used for artificial insemination because they had very low TM and PM. The inseminating dose was 100 million spermatozoa.

Artificial insemination (AI) was performed between 50 and 56 h after the removal of the intravaginal progesterone implant. After solid and liquid fasting for 12 h the ewes were sedated with 0.05 mL/kg of acepromazine then the ewe was placed on Trendelenburg position. Uterine horns were observed with a 5 mm and 30° angulated laparoscope (Karl Stroz, Tuttlingen, German). The spermatozoa were deposited into the uterine lumen in the middle of the uterine horn using an Robertson pipet (Minitube Brasil, Porto Alegre, Brazil) [24]. Gestation diagnosis was performed by transabdominal ultrasound at 60 days after AI with a 5 mHz linear probe (DP2200 VET, Mindray Medical International Limited, Shenzhen, China).

2.6. Statistical analysis

Analysis of the samples were of paired order and the data were analyzed in randomized block design with six (AV, G0, G6, G12, G24 and G48) or two (CM, SP) groups and ten (AV) or five (G0, G6, G12, G24 and G48) treatments. For the analysis of the data the averages of the values obtained in each treatment were used. The variables showed normal distribution and homogeneity of variances by the Kolmorogov-Smirnov method, being used as statistical methods ANOVA, followed by the Tukey test in the analyzes of the groups and Fisher's exact test in the fertility trial analysis. The statistical program used was Action® version 2.6.216.366, with a significance level of 5%.

3. Results

3.1. Fresh analysis

Total motility of AV group was 86.4 ± 4.9^a and did not differed from G0 (79.0 ± 13.7^a), G6 (81.3 ± 11.3^a) and G12 (72.5 ± 4.6^a). TM of the semen collected from the cauda epididymis decreased ($P < 0.05$) on G24(70.0 ± 5.4^b) and had lower value on G48(39.0 ± 24.2^c). PM was only different ($P < 0.05$) in the G48 (14.0 ± 12.7^b) when compared to semen collected in the artificial vagina (73.2 ± 6.5^a) or any other groups (G0: 62.0 ± 14.0^a; G6: 65.0 ± 9.3^a; G12: 31.3 ± 11.3^a; G24: 26.3 ± 9.2^a). There was no difference in the percentage of cells with acrosomal defects ($P > 0.05$) in any groups, but the G24(34.4 ± 20.9^a) had higher ($P < 0.05$) concentrations of tail defects when compared to other groups(AV: 13.8 ± 8.4^b; G0: 10.0 ± 5.2^b; G6: 16.6 ± 6.2^b; G12: 23.5 ± 16.7^b; G48: 27.6 ± 21.9^b).

3.2. Spermatic recovery

Sperm volume collected in the artificial vagina (1.1 ± 0.2 mL) was significantly lower when compared to samples collected from the cauda epididymides (G0 = 3.1 ± 0.7; G6 = 2.6 ± 0.3; G12 = 3.0 ± 0.4; G24 = 2.8 ± 0.5 mL), while the volume of sperm recovered from the cauda epididymides at 48 h (G48 = 1.9 ± 0.6 mL) post mortem was lower ($P < 0.05$) when compared to other groups. Total spermatozoa recovered were higher in groups G0 ($6936{,}6 \pm 1551{,}4 \times 10^6$), G12 ($6349{,}2 \pm 1009{,}2 \times 10^6$) and G24 ($4239.75 \pm 2491.2 \times 10^6$) when compared to G48 ($2064.2 \pm 1379.3 \times 10^6$) and AV ($2623.6 \pm 1063.4 \times 10^6$).

3.3. Post thaw analysis

Total motility, progressive motility, percentages of viable, damaged acrosome and unstable membrane cells spermatozoa are shown in Table 1. TM declined ($P < 0.05$) at G48. PM were higher ($P < 0.05$) in AV group when compared to samples collected from epididymis. G48 had the lowest ($P < 0.05$) percentage of PM and viable cells when compared to other groups of samples collected from the epididymis, but did not differ from the AV group. The highest ($P < 0.05$) incidence of cells with damaged acrosomes was identified in group G48. The percentage of unstable membrane cells did not differ in any of the groups evaluated (Table 3).

ALH, BCF, STR and LIN did not differ between groups AV, G0, G6, G12 and G24, but lower values ($P < 0.05$) were seen in G48 when compared to other groups. VSL was superior in AV group when compared to all groups of samples from the cauda epididymis. VAP and VCL of AV and G0 groups did not differ from each other and were higher ($P < 0.05$) when compared to the G6, G12, G24 and G48 groups, and G48 had a lower VAP ($P < 0.05$) when compared to all other groups (Table 2).

Table 1
Mean ± standard deviation of TM (%), PM (%), viable (%, YOPRO-), damaged acrosome (%, FIT-PSA+) and unstable membrane cells (%, M540+) of samples post thaw collected from ten rams in an artificial vagina (AV) and from the cauda of 4 epididymis each group at 0(G0), 6(G6), 12(G12), 24(G24) and 48(G48) hours post mortem.

	TM	PM	Viable	Damaged Acrosome	Unstable membrane
AV	56.3 ± 22.5^{a}	26.8 ± 14.3^{a}	18.9 ± 9.4^{ab}	23.1 ± 8.6^{a}	2.7 ± 2.9^{a}
G0	34.1 ± 26.9^{ab}	11.7 ± 9.8^{b}	15.4 ± 9.2^{a}	30.67 ± 9.7^{a}	2.4 ± 1.9^{a}
G6	54.6 ± 20.9^{a}	13.1 ± 7.9^{b}	24.5 ± 8.3^{a}	25.5 ± 9.7^{a}	1.5 ± 1.3^{a}
G12	38 ± 23.2^{ab}	3.1 ± 3.8^{bc}	21.4 ± 11.3^{a}	27.0 ± 12.1^{a}	3.1 ± 2.1^{a}
G24	28.6 ± 17.9^{ab}	2.1 ± 1.7^{bc}	22.7 ± 11.9^{a}	30.8 ± 17.7^{a}	2.9 ± 2.4^{a}
G48	6.1 ± 7.0^{b}	0 ± 0^{c}	5.1 ± 5.5^{b}	57.1 ± 18.6^{b}	0.9 ± 0.5^{a}

Different letters in the same column indicate significant difference (P < 0.05) between results.

3.4. Evaluation of extenders and fertility trial

Samples collected in artificial vagina cryopreserved with CM had higher (P < 0.05) TM than G48 cryopreserved with CM or SP. TM did not differ in any other group. PM was higher (P < 0.05) in AV group cryopreserved with CM when compared to all other groups. G6 CM had higher (P < 0.05) viability when compared to G48 SP but did not differ from other groups. G48 had higher (P < 0.05) damaged acrosome when compared to AV group cryopreserved with CM and G6 cryopreserved with SP. Unstable membrane spermatozoa did not differ between any groups. The only difference observed between cryopreservation extenders was on PM between AV groups (Table 3).

Number of pregnant and inseminated ewes and pregnancy rates after IA by laparoscopy using post thaw semen collected in AV and from the cauda epididymis kept at room temperature for 0, 6, 12 and 24 h and frozen in CM and SP are shown in Table 3. Although gestation rates varied in their absolutes values, there was no difference between groups and extenders when statistical tests were performed. None ewe of G0 SP and G24 CM groups were pregnant on ultrasonographical exam.

4. Discussion

The current study evaluated the maximum period of exposure of ovine epididymides to room temperature post mortem after which viable spermatozoa could still be collected and frozen.

In the present study, post-thawing results showed that epididymal spermatozoa collected immediately after death yielded total motility, viability, damaged acrosome and unstable membrane cells values equal to ejaculated samples. Other authors reported that spermatozoa from cauda epididymis can be frozen with same or better post thaw results than ejaculated semen [4,25]. TM from samples collected from the epididymides post mortem did not differ from those in the AV group up to 24 h post mortem. The results of this study were similar to those reported by other authors working with bulls [3] and sheep [1,25,26]. Epididymal samples PM were lower (P < 0.05) when compared to the AV group, we hypothesized that this result is due to high numbers of distal cytoplasmic droplets in the tail of the epididymal spermatozoa frequently observed at CASA monitor. Bertol et al. [27], also reported a large number of distal and medial cytoplasmic droplets in cryopreserved spermatozoa from bull epididymides. According to Ehling et al. [28], cytoplasmic droplets are not abnormal due to the origin of the spermatozoa. The length of time that epididymides were kept post mortem before sperm collection affected PM corroborated findings of other authors [8]. Martinez-Pastor et al. [9] working with wild ruminants, reported an increase of osmolality as a reflect of epidydimal histological changes and a moderate negative correlation between TM and PM and the time post mortem after which semen is collected from cauda epididymis. Songsasen et al. [29] described that degeneration of epididymal tubules starts within 18 h of death in rats. Decrease in TM and PM found in our study is in agreement with other authors who associated sperm depreciation over post-mortem time with epididymal cells autolysis and alterations in epididymal environment [5,9,30,31].

VAP and VCL did not differ between AV and G0 groups, but decreased (P < 0.05) gradually from G6. VSL was lower (P < 0.05) in all groups when compared to the AV group. VAP, VSL, VCL, ALH, BCF, STR and LIN were lower in G48 (P < 0.05) than all other groups. According to Nichi et al. [32] when epididymides are exposed to higher temperatures prior to spermatozoa cryopreservation the mitochondrial membrane potential decreases significantly. We speculate that the exposure of the epididymides to room temperature changes the movement pattern of spermatozoa, meaning that spermatozoa migrate more slowly through female reproductive tract. Cox et al. [33] studied the sperm kinetics of goat semen and showed that ejaculates with higher values of VCL, VAP and VSL were more efficient in the cervical mucus migration test. However, studies in man [34], sheep [35], stallions and bulls [36] found no relationship between kinetic parameters evaluated in CASA and fertility.

Plasma and acrosomal membrane appear to support better post mortem conditions [37] when compared to other parameters of sperm quality, such as motility and kinetics. Some authors [4,25] have hypothesized that epididymal spermatozoa could be resilient to thermal shock and osmotic changes and, consequently, to cryopreservation. This hypothesis was not proven in the present study because there was no difference in viability and numbers of damaged acrosomes between AV and G0 groups (Table 3). Viability

Table 2
Mean ± standard deviation of VAP(μm/s), VSL(m/s), VCL(μm/s), ALH(μm), BCF(Hz), STR (%) E LIN (%) of samples post thaw collected from ten rams in artificial vagina (AV) and from the cauda of 4 epididymis each group at 0(G0), 6(G6), 12(G12), 24(G24) and 48(G48) hours post mortem.

	VAP	VSL	VCL	ALH	BCF	STR	LIN
AV	95.6 ± 13.5^{a}	76.2 ± 12.5^{a}	187.0 ± 22.7^{a}	8.2 ± 0.4^{a}	38.5 ± 2.3^{a}	75.3 ± 3.1^{a}	39.7 ± 1.5^{a}
G0	74.7 ± 10.3^{a}	57.1 ± 5.9^{b}	159.7 ± 18.0^{ab}	7.6 ± 0.7^{a}	39.6 ± 4.0^{a}	72.5 ± 4.5^{a}	35.3 ± 2.4^{a}
G6	65.4 ± 11.0^{b}	49.3 ± 9.1^{bc}	132.6 ± 19.7^{b}	7.2 ± 0.5^{a}	37.4 ± 2.5^{a}	72.4 ± 6.3^{a}	38.1 ± 4.4^{a}
G12	54.6 ± 9.5^{b}	35.2 ± 7.5^{c}	121.4 ± 15.2^{b}	6 ± 2.8^{a}	34.8 ± 2.24^{a}	65.3 ± 6.2^{a}	32.6 ± 4.2^{a}
G24	57.6 ± 6.8^{b}	37.9 ± 7.9^{c}	122.1 ± 9.2^{b}	6.5 ± 2.1^{a}	34.3 ± 3.8^{a}	65.5 ± 4.1^{a}	33.0 ± 3.0^{a}
G48	29.3 ± 27.6^{c}	14.7 ± 12.6^{d}	66.2 ± 59.4^{c}	0 ± 0^{b}	20.1 ± 18.5^{b}	33.6 ± 28.5^{b}	15.5 ± 14.4^{b}

Different letters in the same column indicate significant difference (P < 0.05) between results.

Table 3
Mean ± standard deviation of TM (%), PM (%), viable (%, YOPRO-), damaged acrosome (%, FIT-PSA+), unstable membrane cells (%, M540+), number of pregnant and inseminated ewes and pregnancy rate of samples cryopreserved with control media (CM) and CM plus 20% of seminal plasma (SP) collected from ten rams in artificial vagina (AV) and from the cauda of four epididymis each group at 0(G0), 6(G6), 12(G12), 24(24) and 48(G48) hours post mortem.

		TM	PM	Viable	Damaged Acrosome	Unstable membrane	Pregnant/Inseminated	Pregnancy rate
AV	CM	75.5 ± 10.0^{a}	37.3 ± 11.5^{a}	12.3 ± 1.3^{ab}	18.9 ± 4.3^{b}	1.4 ± 0.9^{a}	2/13	15.4^{a}
	SP	37.0 ± 6.55^{abc}	16.3 ± 7,0^{b}	20.1 ± 8.2^{ab}	27.3 ± 10.5^{ab}	4.0 ± 3.9^{a}	2/13	15.4^{a}
G0	CM	37.5 ± 33.9^{abc}	13.0 ± 11.5^{b}	13.0 ± 2.0^{ab}	35.0 ± 6.3^{ab}	3.8 ± 2.4^{a}	2/7	28.6^{a}
	SP	31.4 ± 23.5^{abc}	10.8 ± 9,5^{b}	17.2 ± 11.8^{ab}	36.9 ± 19.6^{ab}	1.9 ± 1.1^{a}	0/10	0.0^{a}
G6	CM	58.5 ± 19.3^{ab}	15.25 ± 9.0^{b}	23.4 ± 8.8^{a}	32.1 ± 7.1^{ab}	2.2 ± 1.5^{a}	2/7	28.6^{a}
	SP	50.7 ± 24.7^{abc}	11.0 ± 7.0^{b}	22.5 ± 7.9^{ab}	18.8 ± 7.1^{b}	0.7 ± 0.5^{a}	2/7	28.6^{a}
G12	CM	44.25 ± 19.9^{abc}	3.0 ± 3.4^{b}	16.65 ± 10.7^{ab}	34.3 ± 8.0^{ab}	4.4 ± 2.2^{a}	1/8	12.5^{a}
	SP	29.6 ± 28.8^{abc}	3.3 ± 4.9^{b}	19.8 ± 13.5^{ab}	19.5 ± 11.2^{b}	1.8 ± 1.0^{a}	4/10	40.0^{a}
G24	CM	33.25 ± 24.1^{abc}	2.75 ± 2.3^{b}	17.8 ± 4.8^{ab}	30.4 ± 11.1^{ab}	3.15 ± 3.3^{a}	0/10	0.0^{a}
	SP	24.0 ± 10.5^{abc}	1.5 ± 0.5^{b}	21.8 ± 15.0^{ab}	31.2 ± 24.6^{ab}	2.5 ± 1.3^{a}	3/10	30.0^{a}
G48	CM	10.6 ± 9.7^{bc}	0.0 ± 0.0^{b}	1.9 ± 0.8^{b}	51.2 ± 19.8^{a}	0.4 ± 0.2^{a}	–	–
	SP	3.0 ± 4.24^{c}	0.0 ± 0. 0^{b}	6.6 ± 6.5^{ab}	59.0 ± 19.7^{a}	1.2 ± 0.5^{a}	–	–

Different letters in the same column indicate significant difference ($P < 0.05$) between results.

and acrosome integrity decreased ($P < 0.05$) in G48, probably due to the degeneration of the seminiferous tubules, increased osmolarity and pH [26] that occur post mortem. Kaabi et al. [1] also observed an increase in numbers of damaged acrosomes 48 h after death in thawed ovine epididymal spermatozoa.

When sperm is deposited in the female reproductive tract, spermatozoa undergo structural and metabolic changes, called capacitation [38]. Merocyanine 540 can be used to monitor the level of scrambling of the plasma membrane lipid bilayer's phospholipids [22], which can be used to indicate an early stage of sperm capacitation [39]. Some authors have reported that spermatozoa collected from the cauda epididymis of stallions are incapable (or less capable) of undergoing capacitation [10]. In contrast, Okazaki et al., 2012 concluded that boar epididymal spermatozoa have more capacitation when compared to ejaculated semen. In the present study, there was no difference ($P > 0.05$) in the incidence of unstable membrane spermatozoa between the AV group and epididymal samples (Table 3). However, no protocol to sperm capacitation was used.

Addition of seminal plasma to cryopreservation extender had no effect on any seminal parameter nor fertility. The exception was AV group, that had higher ($P < 0.05$) PM in samples cryopreserved with CM when compared to SP, probably due to individual effect since AV semen already owns natural seminal plasma. The absence of seminal plasma effect differed from findings of other authors who added seminal plasma to the diluting medium for sperm cooling [19,40], freezing extender [41,42] or evaluated fertility [19]. In contrast, other authors [43,44] reported that the addition of seminal plasma to cryopreserved semen did not alter fertility after cervical or intrauterine insemination in sheep. These various effects are probably the result of the great variability of organic and inorganic components found in seminal plasma of sheep [44]. The composition of seminal plasma and its effect on semen quality and fertility may vary from species to species [45], by semen collection method [46], individual effect [47] and, in sheep, according to the season [48]. We hypotheses that the seminal plasma collection in summer may had affected our results. Domínguez et al. [12] reported that seminal plasma protein concentration were higher in autumn (31 mg/mL) compared to winter, spring and summer (21, 10.5, and 21.2 mg/mL respectively).

Pregnancy rate after laparoscopic insemination was lower than reported by other authors after laparoscopic insemination with ovine epididymal spermatozoa [25] or ovine ejaculated semen [25,43,49]. It is possible that the lag period of insemination (end of breeding season), the high number of young ewes and the sample size of inseminated ewes by groups may have contributed to these results. There was no statistical difference in pregnancy rate after laparoscopic artificial insemination between AV and cauda epididymis groups (Table 3). This result corroborates findings of Monteiro et al. [4] who found similar pregnancy rates between mares inseminated with sperm recovered from the cauda epididymis and with ejaculated sperm. Álvarez et al. [25] also reported similar sheep fertility after laparoscopic insemination with ejaculated, electro ejaculated and sperm from the cauda epididymis. Some authors have described that the fertility of ovine epididymal spermatozoa decreases by 48 h post mortem when the epididymides are kept refrigerated [1]. According to Abella et al. [50] most of the epidydimal spermatozoa, which remain motile, also retain their fertilizing ability. In our study all samples tested in fertility trial remain with a minimum TM of 24%, consequently all samples tested remain with minimum number of thawed motile spermatozoa which still yield satisfactory fertility [51,52].

In conclusion, measured parameters, except for PM and VSL, were no different between ovine spermatozoa from the cauda epididymis and ejaculated sperm, under the conditions of our study. It is possible to cryopreserve spermatozoa from the cauda epididymis that have been exposed to room temperature for up to 24 h post mortem without loss of viability or fertility. Finally, the addition of 20% seminal plasma to the cryopreservation extender had no effect on sperm viability or fertility.

Acknowledgements

T.G. Bergstein-Galan was supported by scholarship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES (Brazil).

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